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Rudimentary form of cellular "vision"

(BHK cells/infrared light/cell polarity/cell–cell communication)

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ABSTRACT **BHK cells were inoculated sparsely on one face ("sparse- or s-face") of a thin glass film whose opposite face was covered with a 2- to 3-day-old confluent layer of BHK cells ("confluent- or c-face"). After 7 hr of attaching and spreading in the absence of visible light, most of the cells on the s-face traversed with their long axes the direction of the whorls of the confluent cells on the c-face directly opposed. The effect was inhibited by a thin metal coating of the glass films. The results suggest that the cells were able to detect the orientation of others by signals that penetrated glass but not thin metallic films and, therefore, appeared to be carried by electromagnetic radiation. In contrast, the effect was not influenced by a thin coat of silicone on the glass, suggesting that the wavelength of this radiation is likely to be in the red to infrared range. The ability of cells to detect the direction of others by electromagnetic signals points to a rudimentary form of cellular "vision."**

A previous article had suggested among other possibilities that 3T3 cells located and tried to approach distant infrared light sources because they mistook them for other cells (1). If this were true, mammalian tissue cells should be able to detect each other across a partition made of glass.

To test this possibility, I studied the behavior of BHK cells that were plated on opposite faces of thin glass films. Cells were plated densely (confluent layer) on one face ("c-face") of the glass film and were allowed to form their typical patterns of whorls. Two days later cells were plated sparsely on the opposite face ("s-face") and were kept in total darkness in a culture incubator for 7 hr while they attached and spread.

The cells of the second inoculum did not spread randomly but oriented themselves with respect to the cells directly opposed on the other face of the glass film. The present article describes these experiments and others which suggest that the most likely explanation for this phenomenon involves the processing of electromagnetic signals by the cells.

MATERIALS AND METHODS

Glass Films. The experiments used soda-lime glass substrates with four different levels of thickness. (*i*) Thin glass films were produced by cutting off the tip of a Pasteur pipette and melting the stump in the flame of a Bunsen burner until it assumed a conical shape. After rapid removal from the flame, the tip was blown into a 6- to 10-cm-diameter balloon by a 1.2-s-long burst of compressed air at 2.5 psi (1 psi = 6.89 kPa). The burst was controlled by a digital time switch (Gralab 451; Thomas Scientific) that opened a solenoid. In areas where the balloon showed strong interference colors, its wall thickness had decreased to <2 μm as determined by scanning electron microscopy with several different samples. These areas of the balloon were broken off, and fragments larger than 5 × 15 mm^2 were collected. (*ii*) Regular glass films came from the other areas of the glass balloon that had wall thicknesses of 6–8 μm as determined by scanning electron microscopy. They were fragmented and collected in similar ways. (*iii*) Thin glass plates 30–40 μm thick were generated by cutting a 12 × 50 mm^2 strip from normal 24 × 50 mm^2 coverslips of no. 1 thickness (130–160 μm), mounting it vertically, and attaching a weight of 8 g to its lower end. After its central portion was heated with a Bunsen burner, the weight stretched it and produced 30- to 40-μm-thick plates as determined by face-on views in the light microscope. (*iv*) Normal no. 1 coverslips (thickness of 130–160 μm) were used as "thick" control substrates.

Regular Test Substrates. Fragments of the glass film were glued with nontoxic epoxy resin (Elmer's Clear Epoxy, Borden, Columbus, OH) over 2 × 10 mm^2 slits in normal no. 1 glass coverslips that had been drilled with a high-speed dental drill (see Fig. 1*a Inset*). Subsequently, BHK cells (3- to 6-day-old cells; passage interval, 2–3 days; passage ratio, 1:5; passage numbers, 12–20) were plated densely (five drops of 3,000,000 cells per ml into a 35-mm dish) onto one face (c-face) of the test substrate and were allowed to grow to confluence in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) calf serum at 37°C in an atmosphere containing 8% CO_2 and saturated humidity. After 2 days the test substrate was turned upside down, and new BHK cells were plated sparsely (1- to 3-day-old cells; two drops of a single-cell suspension of 2,000,000 cells per ml into a 35-mm dish) on the new upper face (s-face). The dishes with the test cells were kept for 7 hr in a light-tight incubator before they were fixed in 3.7% formaldehyde in phosphate-buffered saline for 10 min. Subsequently, Coomassie blue solution was added to the dishes while the s-face of the test substrates was pointing up. In this way the cells on the s-face were stained with Coomassie blue while the cells on the c-face remained practically unstained. After the test substrates were washed in distilled water, they were mounted on glass slides.

Test Substrates with Orienting c-Faces ("Orienting Substrates"). To reduce the visual impression of randomness of the cell orientations, I carried out a separate series of experiments using specially prepared substrates with orienting c-faces. When inoculated on the c-face of these substrates, the cells were preferentially lined up along parallel stripes in the following way. After the c-face of the glass film was coated with a plastic that was poorly adhesive for the cells, a pattern of alternating metallic and transparent parallel stripes was printed by vacuum evaporation of an alloy on the plastic coat, thus creating a surface to which the cells could adhere quite strongly. The plastic coating that was poorly adhesive for cells was produced by spraying a solution of 10 mg of polystyrene (fragments of Corning plastic culture dishes) per ml of amyl acetate from a distance of 15 cm. The coats were immediately dried with hot air from a hair dryer and yielded values for their thickness of <0.5 μm. To produce the striped metallic patterns, several electron-

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Abbreviations: c-face and s-face, faces of a slide covered with a confluent layer and a sparse layer of cells, respectively.

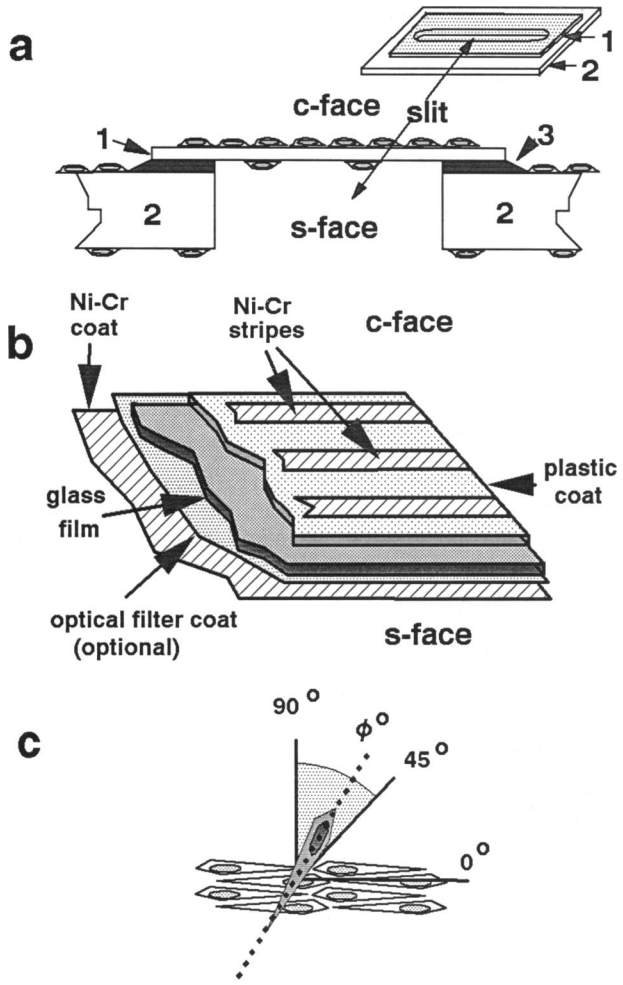

FIG. 1. Basic designs. (*a*) Regular test substrates. 1, thin glass film; 2, normal coverslip; 3, epoxy layer that glues the glass film over the slit in the coverslip. (*b*) The various layers that constituted an orienting test substrate. The thicknesses are not drawn to scale. (*c*) Definition of the traversing angle ϕ of a cell on the s-face (shaded cell) relative to the collective direction of whorls of cells on the c-face. A cell on the s-face is considered as traversing when $\phi \geq 45°$ (stippled sector).

microscope grids (300-mesh parallel-line copper grids; Ted Pella, Tustin, CA) were attached to the plastic film by tiny spots of silicone high-vacuum grease (Scientific Products). Subsequently, the test substrates were placed in a DV-502 vacuum evaporator (Denton Vacuum, Cherry Hill, NJ) and ≈10 mg of Chromel A wire (78.4% Ni/20% Cr/1% Si/0.5% Fe; Ogden, Arlington Heights, IL) was melted in a tungsten wire basket and evaporated to form a deposit on the plastic-coated c-face of the test substrates at pressures below 2 μtorr (1 torr = 133.3 Pa) and currents of 15 A for 2–3 min from a distance of 18 cm. The grids were then removed, leaving on the plastic coat unmetalized shadows alternating with metallic coats. The deposited metallic layers were calculated to be no thicker than 30 Å. Their optical transmission was measured with a Beckman DU-7 spectrophotometer and was found to be uniformly 85–90% in the range of wavelengths between 400 and 900 nm. Subsequently, a similar thin Ni–Cr layer was evaporated over the entire s-face to ensure that the cells on both faces were exposed to the same Ni–Cr surface material (Fig. 1*b*).

The Ni–Cr alloy was chosen because the evaporated metal coats did not detach upon contact with culture medium. Furthermore, BHK cells on the deposited Ni–Cr surfaces expressed normal spreading behavior and morphology. BHK cell cultures grown on the deposited Ni–Cr surfaces for 1 day were indistinguishable from control cultures on glass. However, after 2 days they had grown 46% less than the control cultures, although they did not contain any dead, floating cells.

Orienting Test Substrates Coated with Optical Filters. To test the role of electromagnetic radiation in the described experiments, 100- to 150-Å-thick highly absorbing layers of Ni–Cr or silicone were added to the s-face of orienting substrates [labeled "optical filter coat (optional)" in Fig. 1*b*]. The Ni–Cr filters were generated by evaporating 30 mg or more of Chromel A wire from a distance of 7–8 cm. The resulting optical transmissions were uniform with values of 10% to 0.4% in the 400- to 900-nm-wavelength range (examples of the absorption spectra are shown further below in Fig. 5*b*).

Similarly, 17–20 mg of silicone (Aldridge) were evaporated 7–8 cm away, resulting in dark-brown coats whose absorption decreased monotonously between 400 nm and 900 nm. To prevent detachment of the silicone coats, it was necessary to first evaporate a very thin Ni–Cr undercoating as described above. A similarly thin Ni–Cr coat was also placed on top of the silicone surface to standardize the surface material to which the cells were exposed.

Evaluation of the Results. *Definition of "traversing" cells.* Ignoring the front–rear polarity of the cells, the crossing angle ϕ between the long axis of a cell on the s-face (Fig. 1*c*; shaded cell) and the direction of whorls of cells on the c-face (marked 0° in Fig. 1*c*) was defined as an angle in the interval between 0° and 90°. Cells on the s-face were defined as "traversing" when the crossing angle $\phi \geq 45°$ (Fig. 1*c*; stippled sector).

Seven hours after the inoculation of cells on the s-face, the number of traversing cells was counted and the percentage, p_+, was calculated from $p_+ = 100 \cdot (N_+/N)$, in which N is the total number of counted cells on the s-face and N_+ is the number of traversing cells among them. The specific time point of 7 hr was chosen because at this time practically all BHK cells had spread, while relatively few of them had started to migrate.

Cells were excluded from the counts when they (*i*) had no obvious long axis (e.g., round, branching, or strongly curved cells), (*ii*) were located directly opposed to areas of criss-crossing cells on the c-face, or (*iii*) were located adjacent to small cracks or edges in the glass film (because such locations could contact-guide the test cells regardless of the orientation of the c-face cells).

The subjectivity of the counts was tested by asking three test persons to score blindly two preparations. Their counts remained within 5% of my counts.

Under these conditions the random expectation of the percentage of traversing cells on the s-face is $p_+ = 50\%$ because half of a randomly oriented population of cells must form crossing angle $\phi \geq 45°$, while the other half forms $\phi \leq 45°$.

Statistical evaluation. Two methods were used for statistical treatment of the results. One method evaluated each preparation individually and determined the average and standard deviation of the data of repeated experiments. The second method evaluated the cumulative counts for the different experimental conditions. The two methods showed effectively the same results.

Determination of the distributions of crossing angles. Since the cells on the c-face of orienting substrates could be considered as having a fixed orientation, the experimental error of the crossing angle was half as large as in the case of regular isotropic substrates. Therefore, orienting substrates were used to determine the distribution of crossing angles. For the measurements the microscopic image of the preparations was displayed on a television monitor with the stripes of oriented cells on the c-face parallel to the horizontal axis.

By using a transparent protractor with 30° divisions, the crossing angles were sorted into histograms with the three sorting intervals of 0–30°, 30–60°, and 60–90°. Although the orientation of the cells on the c-face showed deviations from the direction of the stripes, the influence of these deviations could be averaged out by counting large samples of cells. Therefore, for each set of experimental parameters, a total of 550–900 cells were counted in four to seven different preparations. It also was important for the objectivity of the counts to remove optically the phase-contrast image of the stripes of cells on the c-face and display only the stained cells on the s-face by using normal bright-field light microscopy.

RESULTS

A typical result with regular nonorienting test substrates is shown in Fig. 2. It appeared that the long axes of the cells on the s-face were predominantly oriented in a traversing direction relative to the pattern of whorls of the confluent cells on the c-face (e.g., white frame in Fig. 2). The observation suggests that the cells interacted with each other across the glass partition. To explain the local character of this previously unreported form of cell–cell and cell–environment interaction, it seems inevitable to postulate the existence of certain cellular and environmental signals that pass directly through the glass and carry information about the orientation of the cells.

Inhibition by Increasing Thickness of the Glass Film. Counting between 556 and 984 cells from 5–13 different preparations, I found that 76% (SD = 4%) of the cells on the s-face oriented their long axes in a traversing direction relative to the direction of the whorls on the c-face. However, increasing the film thickness from 1–2 μm to 160 μm reduced the percentage of traversing cells to the level expected of random orientation (Fig. 3). The percentages of traversing cells were the same whether they were calculated as the averages of individual ratios of counts or as the ratio of the cumulative counts. (Table 1.)

Angular Distribution of Crossing Angles. The measurement of the angular distribution of the crossing angles required the definition of sorting intervals that were larger than the experimental error. To justify the use of at least three sorting intervals (namely, 0–30°, 30–60°, and 60–90°), it was necessary to reduce the experimental error to a value of 10–15° by lining up the cells on the c-face in a uniform and fixed orientation. The required orienting substrates were produced as described. Fig. 4 shows examples of the stripes of horizontally oriented cells on the c-face of regular glass films of 6- to 8-μm thickness together with the predominantly traversing cells on the s-face 7 and 24 hr after plating. The nonrandom orientation of the traversing cells on the s-face appeared more striking after 24 hr (Fig. 4*b*) than after 7 hr. However, much of their orientation may result from the guidance of newly divided cells alongside other cells that had oriented themselves earlier on the same face. Therefore, such preparations expressed an unknown contribution of cell–cell interaction between cells on the same face and therefore were excluded from the quantitative evaluations. Only the 7-hr preparations were used because at this time the growth of the cells on the s-face could be neglected. The histograms of such preparations express the nonrandom orientation as a rising of the counts with increasing crossing angles (Fig. 5*d*; 880 cells counted).

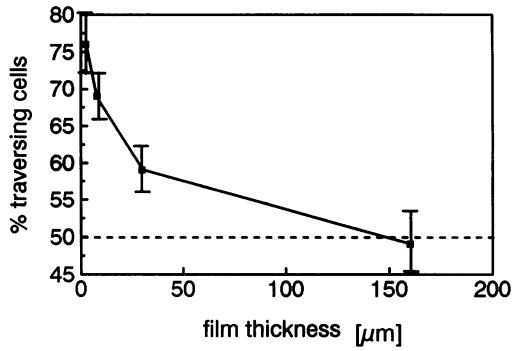

FIG. 3. Percentage of traversing cells on the s-face 7 hr after plating as a function of the approximate thickness of the glass film. Error bars represent standard deviations.

Evidence for Red or Infrared Light as Signal Carrier. The percentage of traversing cells on orienting substrates was computed from the histogram of Fig. 5*d* to be ≈60%. This value was lower than the percentage of traversing cells on regular test substrates of the same glass thickness (69%; see Fig. 3). When one considers that the thin Ni–Cr coats on the orienting substrates absorbed more light than the regular glass films did (Fig. 5*a*), the reduced percentage may be taken as a first hint that light might play a role in the explanation of the effect. To test whether the unknown signals indeed were carried by electromagnetic radiation, layers of Ni–Cr with much higher levels of absorption were deposited on the s-faces of orienting substrates before plating out the test cells. The metal layers were only 100–150 Å thick and thus did not

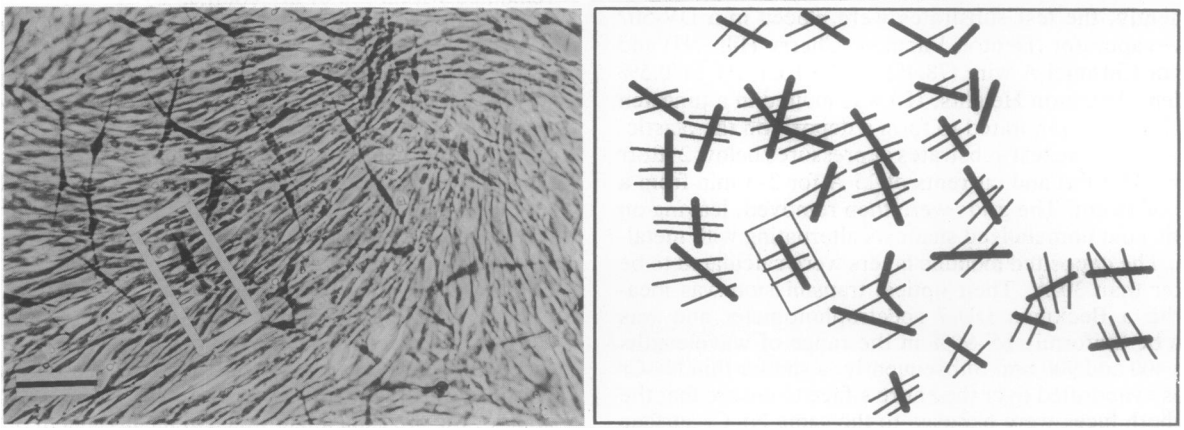

FIG. 2. A field of typical preparation 7 hr after plating BHK cells on the s-face (*Left*) together with a drawing of the micrograph to highlight the orientation of the cells on the s-face (fat lines) and the direction of the cells on the c-face directly opposed (thin lines) (*Right*). The predominant orientation of BHK cells on the s-face (sparse, darkly stained cells) traverses the local direction of whorls of BHK cells on the c-face (unstained, dense layer of cells) seen in phase-contrast. One of the examples of a cell traversing the local direction of the cells on the other face of the glass film is surrounded by a white frame. (Bar = 100 μm.)

Table 1. Evaluation of the percentage $p_+ = 100 \cdot (N_+/N)$ of traversing cells on the s-face of regular test substrates 7 hr after inoculation

Appr. film thickness, μm	Traversing cells in indiv. preps., %	N	N_+	%*	%†
2.5	79, 74, 73, 81, 70	562	427	75 ± 5	76 ± 4
8	73, 66, 71, 76, 69, 65, 70, 66, 70, 68, 68, 68	984	680	69 ± 3	69 ± 3
30	59, 60, 53, 60, 64, 52, 63	556	327	58 ± 5	59 ± 3
160	47, 48, 50, 51	377	184	48 ± 2	49 ± 4

Appr., approximate, indiv. preps., individual preparations; N, total number of cells counted under similar experimental conditions; N_+, total number of traversing cells counted under similar experimental conditions.

*Average percent of traversing cells based on the individual counts shown in the second column ± SD.

†Average percent of traversing cells based on the number N of total cells counted and the number N_t of total traversing cells counted ± $100\sqrt{N_+}/N$, assuming a Poisson-distribution of the traversing cells.

alter significantly the thickness of the already several-micrometer-thick glass film. However, they absorbed visible and infrared light uniformly across the range of wavelengths (Fig. 5*b*). Measurements of the crossing angles of 905 cells on such orienting substrates with optical transmissions ranging from 0.4% to 10% showed a random orientation of the cells on the s-face (Fig. 5*e*). The result suggested that the orienting signals between the two cell populations were blocked by the metal film and thus were likely to be carried by electromagnetic radiation.

The range of wavelengths of this radiation could be narrowed to the red-to-infrared range by using silicone instead of Ni–Cr as the optical filtering material. As shown in Fig. 5*c*, layers of evaporated silicone on the s-face of the glass films absorbed strongly at the blue end of the visible spectrum but became increasingly transparent for red and infrared light. Counting the crossing angles of 547 cells on such substrates (Fig. 5*f*) showed essentially the same distribution as on the normal glass films (cf. Fig. 5*d*), suggesting that the orienting signals were able to pass through the silicone and thus were likely to belong to the red-to-infrared range.

Recognition of Fixed Cells on the c-Face. The results may suggest that the cells on either faces emitted and received cell-specific signals that caused them to orient relative to each other. Therefore, several preparations with cells on the

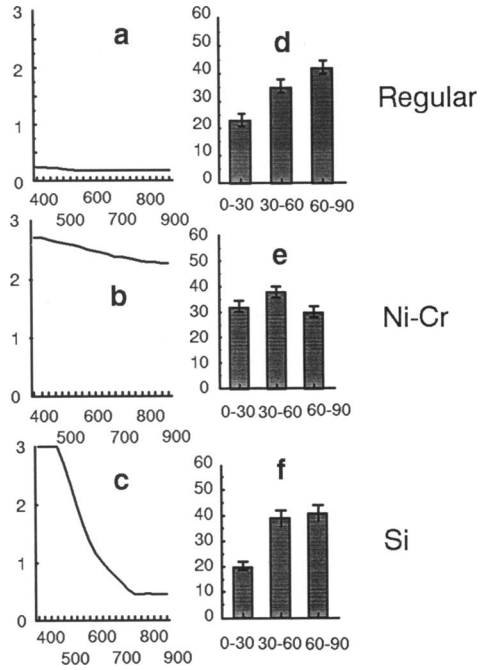

FIG. 5. Absorption spectra of several orienting test substrates with 6- to 8-μm-thick glass films together with the histograms of the crossing angles of BHK cells on these substrates 7 hr after inoculation. Ordinates are absorption units (*a*, *b*, and *c*) and percentage of cells (*d*, *e*, and *f*). Abscissa are wavelength in nm (*a*, *b*, and *c*) and sorting intervals of crossing angles (*d*, *e*, and *f*); bars indicate the standard deviations of the percentages. (*a* and *d*) Regular orienting test substrates (880 cells; six different preparations). (*b* and *e*) Orienting substrates with a 100- to 150-Å-thick film of Ni–Cr (905 cells; six different preparations with optical transmissions ranging from 0.4% to 10%). (*c* and *f*) Orienting substrates with a film of silicone (547 cells; five different preparations).

c-face of orienting substrates were fixed before the test cells were plated on the s-face. To avoid residual fixative in the preparations, a volatile fixative was used. After incubating the cells for 20 s at −20°C in 1:1 (vol/vol) methanol/acetone, the salt precipitates were removed by a quick wash in distilled water. Subsequently, the preparations were dipped again into the fixative for a few seconds and then air-dried on a warm plate to allow the fixative to evaporate. Finally, the preparations were returned to their original culture medium and

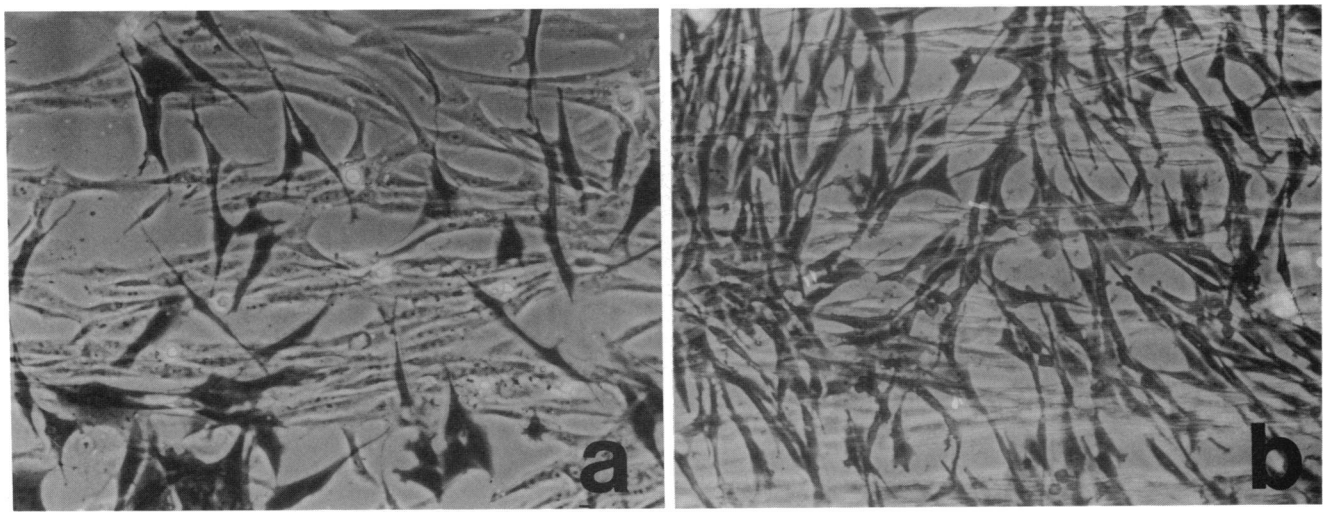

FIG. 4. Cells on the s-face (stained) traverse the long axes of oriented cells on the c-face 7 hr (*a*) and 24 hr (*b*) after inoculation of the cells on the s-face.

inoculated with the test cells. The results were essentially identical to the case of live cells on the c-face, suggesting that the cells were able to "recognize" the orientation of inanimate structures on the c-face.

DISCUSSION

Hypothesis of Electromagnetic Radiation as Carrier for the Orienting Signals. At first it may seem surprising that the BHK cells oriented themselves in a traversing rather than a parallel direction relative to the cells directly opposed. However, it should be noted that traversing cells can frequently be seen in confluent cultures of BHK cells. Furthermore, there are several kinds of connective and other tissues that express a similar "fabric-weaving" or "force-distributing" tendency of other cell types *in vivo*, including cells in the cornea (2), bone, and intestines. Therefore, it may be quite natural for cells to line up in parallel orientation with others that belong to the same cell layer but to traverse the cells in a layer above and below.

Hence, the main concern of the discussion is how the test cells could have "known" the orientation of the cells on the opposite face of the glass. It seems necessary to conclude that certain kinds of signals passing directly through the glass allowed the test cells to assess the orientation of the cells directly opposed. These signals are still entirely unknown, but the following arguments suggest that they are carried by electromagnetic radiation because (*i*) the material properties of the glass film and the surrounding culture medium seem to exclude every other possibility, and (*ii*) my previous studies (1) had suggested on the basis of entirely different experiments that 3T3 cells were able to locate sources of infrared radiation.

Exclusion of chemical and electrical signals. The types of potential signal carriers that may initially come to mind are chemical signals, electrostatic fields, current fields, or pH gradients that may pass through glass. However, they can be excluded. Obviously, the glass film does not permit chemical signals to penetrate. Galvanic electrical currents and electrostatic fields are also excluded because the resistivity of the soda–lime glass used is $\rho \approx 10^{11}\ \Omega\cdot$cm (3). Therefore, the 2 μm $\times$ 10 mm $\times$ 6 mm^2 large glass films used have a resistance of $R = 3 \times 10^9\ \Omega$. Even if the cells were able to generate a significant voltage difference across the glass film, the resulting current would flow along the film surface and not across the glass because the surrounding culture medium is highly conductive and because the test substrates were surrounded by medium from all sides. The pH-buffering capacity of the surrounding culture medium would also preclude the maintenance of any pH gradients across the glass.

Exclusion of sound waves, magnetostatic fields, stress lines, and local bending of the glass film as carriers. There are several other, although unlikely candidates for a signal carrier. Sound waves or magnetostatic fields generated by the cells might transmit information about the orientation of the cells across the glass. Alternatively, it is conceivable that the glass film contained orienting stress lines or that the cells on the c-face bent the glass film locally and thus generated orienting stress lines or curvatures. However, all of these remote possibilities are excluded by the finding that a metal film as thin as 100–150 Å inhibited the effect. Obviously, such a metal film would not absorb sound waves, shield magnetic fields, neutralize stress lines, or flatten local curvatures of the glass films.

Therefore, I conclude that the unknown cellular or environmental signals are carried by electromagnetic radiation as the only remaining candidate. The finding that a silicone layer in contrast to an absorbing metal film did not inhibit the interaction between the two cell populations is consistent with my earlier report about the ability of 3T3 cells to locate infrared light sources of 800- to 900-nm wavelength (1).

Potential sources of the electromagnetic signals. The sources of the putative electromagnetic signals are not yet known. However, one should keep in mind that the cells and all other objects in their environment at 37°C interact continuously with the natural heat radiation of 310 K. Correspondingly, they are continuously emitting and absorbing infrared light over a wide range of wavelengths with a peak at about 10 μm (4). It is conceivable that the cells on the s-face detected the fixed or live cells on the c-face by differences of the emission and reflection of the heat radiation caused by local differences of their surface geometry and/or materials.

Alternatively, one may think of specific emissions of infrared light by, for instance, the high-energy intermediates inside metabolically active cells which allow the test cells to detect other cells as sources of such emissions. The finding that the test cells were able to detect the orientation of fixed cells seems to exclude this possibility unless one assumes that the test cells illuminated the fixed cells with their own emissions.

Conceptual Implications. Although the sources, specific wavelengths, amplitudes, and duration of the electromagnetic signals are not yet known, we may conclude nevertheless that the test cells on the s-face must have been able to identify and locate at least two different light sources across the barrier of glass. Otherwise, they could not have determined the spatial orientation of the cells on the c-face by electromagnetic signals and responded by adjusting their own orientation. This conclusion is consistent with my earlier report about the ability of 3T3 cells to locate distant microscopic infrared light sources and to distinguish between one and two such sources (1). Therefore, these findings taken together may be interpreted as evidence that the cells possess a rudimentary form of vision and an effector system that enables them to respond to the images of their environment.

This series of experiments started when Antoine Carpentier, a French student at the École Normale Supérieure, Paris, insisted that I should study the behavior of cells that are linked by fiber optics. I am very grateful to Dr. Howard Green (Harvard Medical School) for the many clarifying and encouraging discussions. I should also like to thank my colleagues Drs. James Bartles, Yoshio Fukui, and Alvin Telser for their criticism and moral support, and Dr. John Coleman (Solar Physics, Locust Valley, NY) for making the vital suggestion to use Ni–Cr alloys. The work was supported by grants from the Office of Naval Research (N0014-89-J-1700) and the U.S. Army Research Office (ARO 122-89).

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